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## Motor neuron pathfinding following rhombomere reversals in the chick embryo hindbrain

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### Summary

Motor neurons are segmentally organised in the developing chick hindbrain, with groups of neurons occupying pairs of hindbrain segments or rhombomeres. The branchiomotor nucleus of the trigeminal nerve occupies rhombomeres 2 and 3 (r2 and r3), that of the facial nerve r4 and r5, and that of the glossopharyngeal nerve r6 and r7. Branchiomotor neuron cell bodies lie within the basal plate, forming columns on either side of the ventral midline floor plate. Axons originating in rhombomeres 2, 4 and 6 grow laterally (dorsally) towards the exit points located in the alar plates of these rhombomeres, while axons originating in odd-numbered rhombomeres 3 and 5 grow laterally and then rostrally, crossing a rhombomere boundary to reach their exit point. Examination of the trajectories of motor axons in odd-numbered segments at late stages of development (19-25) showed stereotyped pathways, in which axons grew

laterally before making a sharp turn rostrally. During the initial phase of outgrowth (stage 14-15), however, axons had meandering courses and did not grow in a directed fashion towards their exit point. When r3 or r5 was transplanted with reversed rostrocaudal polarity prior to motor axon outgrowth, the majority of axons grew to their appropriate, rostral exit point, despite the inverted neuroepithelial polarity. In r3 reversals, however, there was a considerable increase in the normally small number of axons that grew out via the caudal, r4 exit point. These findings are discussed with relevance to the factors involved in motor neuron specification and axon outgrowth in the developing hindbrain.

**Key words:** cranial nerve, motor neuron, axon guidance, rhombomere, chick embryo.

### Introduction

During development, the chick embryo hindbrain is transiently divided into segmental units or rhombomeres. The process of segmentation is crucial for the development of this part of the neuroepithelium (reviewed in Keynes and Lumsden, 1990; Lumsden, 1990). Rhombomere boundaries appear in a stereotyped sequence between stage 9 and 12 (Vaage, 1969) and remain clearly visible until around stage 25. Rhombomeres have been shown to be units of cell lineage restriction, based on single cell marking experiments (Fraser et al., 1990). The role of segmentation in the hindbrain region may be to maintain populations of cells with similar range of potentials but different positional identities at successive axial levels. The various cranial nerves, for example, contain neurons with similar functions but with different targets and axonal trajectories.

Early neurogenesis in the hindbrain conforms to a segmental plan, with neuronal differentiation advanced in the even-numbered relative to the odd-numbered rhombomeres (Lumsden and Keynes, 1989). The cranial nerve nuclei are also arranged segmentally. Branchiomotor nuclei lie within pairs of segments, with

their exit point in the anterior rhombomere of the pair. Thus, the trigeminal (V) motor nucleus occupies rhombomere r2 (r2) and r3, with its exit point in r2, while the facial (VII) motor nucleus occupies r4 and r5, exiting in r4, and the glossopharyngeal (IX) motor nucleus occupies r6 and r7, exiting in r6 (Lumsden and Keynes, 1989). The cell bodies of the motor nucleus of the abducens (VI) are also located in a pair of segments, r5 and r6, out of register with the branchiomotor nuclei. The hypoglossal (XII) motor nucleus lies in r8, and the trochlear (IV) nucleus in the anterior portion of r1.

The branchiomotor nerves V, VII and IX supply the motor outflow for the first, second and third branchial arch respectively, so that a pair of rhombomeres corresponds to a single branchial arch. Experiments that mapped the pathways of crest migration from the hindbrain have shown that the neural crest is segregated into streams that colonise individual branchial arches (Lumsden et al., 1991). Crest cells from the r1/r2 region populate the first arch and the trigeminal ganglion, crest from r4 populates the geniculate ganglion and the second arch, and that from the r6 region populates the superior ganglion and the third branchial arch. Some neurons of the trigeminal ganglion are also derived from ectodermal placodes (D'Amico-Martel and

Noden, 1983). Rhombomeres 3 and 5 do not produce any migratory neural crest (Lumsden et al., 1991). The trigeminal, geniculate and superior sensory ganglia lie directly alongside rhombomeres 2, 4 and 6, respectively. Axons of sensory neurons in these ganglia enter the hindbrain in regions of the neuroepithelium that also serve as conduits for the outgoing motor axons. Neural crest cells that give rise to some of the cells of the sensory ganglia may contribute to formation of these exit points.

The highly stereotyped patterning of motor neurons in the hindbrain raises the question of how these neuronal groups are specified, and the characteristic outgrowth patterns accomplished. Branchiomotor neurons, which are the subject of this study, differ from somatic motor neurons of the hindbrain and the spinal cord and from spinal visceral motor neurons. Neurons in the latter categories have a very short pathway inside the central nervous system, with axons exiting the neuroepithelium by penetrating the basal lamina close to their point of origin. By contrast, branchiomotor axons follow a comparatively long internal path, and coalesce to exit the brain in groups via a single, large conduit. Branchiomotor axons within odd-numbered rhombomeres are particularly interesting in this context. Whereas those within even-numbered rhombomeres grow in a more or less straight trajectory from medial to lateral to exit within the same segment, axons within odd-numbered rhombomeres have a longer pathway, growing first laterally and then rostrally to cross a rhombomere boundary and exit in the rostrally adjacent even-numbered rhombomere. The neuroepithelium of odd-numbered rhombomeres may also share cell surface properties, reflected in the fact that r3 and r5 combine without forming a boundary in grafting experiments (Guthrie and Lumsden, 1991).

Retrograde labelling of motor neurons has shown that development in odd-numbered rhombomeres is delayed relative to that in their rostral partners (Lumsden and Keynes, 1989). This suggests that the exit points are established as a pathway for r2 and r4 axons by the time the majority of axons in r3 and r5 start to grow out, and that this region of the neuroepithelium might exert a guiding influence on motor axons. One possibility is that the exit point produces a chemotropic substance that attracts outgrowing motor neurons from a distance. Whether growth cones of branchiomotor neurons exhibit specific responsiveness to attractants emanating from the exit point is not known. Another factor that might contribute to the observed pattern of axon growth might be distributed cues on the neuroepithelium, providing positional information. In the case of odd-numbered rhombomeres, these positional values would have to embody segment polarity. Alteration in the polarity of the neuroepithelial environment would then be expected to result in a corresponding change in axonal trajectories. Also, the floor plate, which lies very close to the column of branchiomotor neuron cell bodies, is known to exert an influence on the development of commissural and motor neurons (Tessier-Lavigne et al., 1989; Yamada et

al., 1991). A repulsive influence on branchiomotor axons might explain at least the initial phase of their outgrowth. Data from other systems suggest that multiple cues are likely to be involved in motor axon guidance (reviewed in Lumsden and Cohen, 1991).

In this study, we address some of the questions surrounding motor neuron development in the hindbrain, first by examining the early patterns of motor neuron differentiation and axon outgrowth. For this we have used whole-mount staining of chick embryos using a monoclonal antibody against a cell surface protein of the immunoglobulin superfamily, SC1/DM1 (Tanaka and Obata, 1984; Burns et al., 1991). We have examined the early trajectories of motor axons in odd-numbered rhombomeres to see how the pattern develops. First, we have investigated the question of whether motor axons navigate towards their intermediate targets (exit points) without making errors. Secondly, we have used donor-to-host transplantation of single hindbrain rhombomeres. Transplants were made using rhombomere 3 or 5, either in the correct orientation as a control, or with their rostrocaudal polarity reversed. After allowing these embryos to develop, the patterning of motor neurons within the transplanted rhombomere was then visualised by retrograde tracing using DiI and DiO.

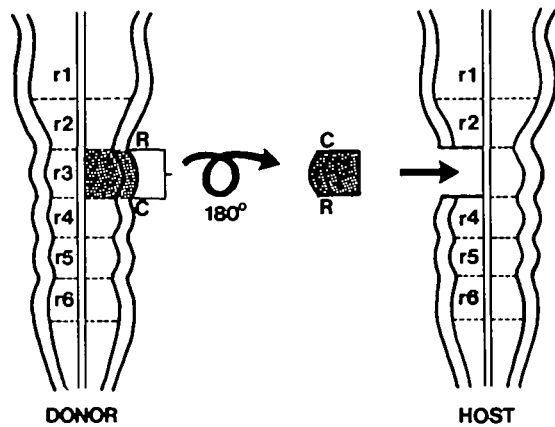
## Materials and methods

### Whole-mount immunohistochemistry

Rhode Island Red hens' eggs were incubated to stages 10-25 (Hamburger and Hamilton, 1951). Embryos were fixed for 2 hours to overnight in 3.5% formaldehyde in PBS, after which the roof plate was cut to allow penetration of antibodies. Embryos were then washed extensively in PBS and blocked overnight in 0.1% hydrogen peroxide in PBS. Embryos were incubated for three days in primary monoclonal antibody DM1 (kind gift of Dr S. Chang) to label motor neurons, followed by exposure to a peroxidase-conjugated secondary antibody (Jackson Immunoresearch, California, USA) overnight and then development using 0.5 mg ml<sup>-1</sup> diaminobenzidine (Fluka) with 0.03% hydrogen peroxide. After washing in PBS, brains were then dissected free of mesenchyme, mounted flat and viewed from the ventral side as flattened whole-mounts using bright-field or Nomarski optics. In some preparations, labelled motor neurons were drawn using a camera lucida.

### Rhombomere reversal experiments

For transplantation experiments, stage 10-12 embryos were used, matching donors and hosts of the same stages. Rhombomeres 3 or 5 were excised from donor hindbrains unilaterally, using needles, flame-sharpened from 100 µm diameter pure tungsten wire. Transverse cuts were made along the rhombomere boundaries at either end, and cuts made longitudinally along the floor plate-basal plate boundary. The underlying pial mesenchyme was left attached to the pieces of neuroepithelium, which were marked with a spot of carmine at their rostral edge. Host embryos were windowed and embryos made visible by sub-blastodermal injection of India ink. Microsurgery was performed through a small opening in the vitelline membrane using tungsten needles. Single rhombomeres were removed from hosts in the same



**Fig. 1.** Diagram showing rhombomere reversal operation. Diagrams of donor and host hindbrains are shown, with rhombomere boundaries indicated by dotted lines. Rhombomere 3 is removed from the right-hand side of a donor stage 10-12 embryo, and rotated through  $180^\circ$  so that its rostrocaudal polarity is reversed (R=rostral, C=caudal). The piece is then inserted into r3 position on the left-hand side of an isochronic host embryo whose r3 has been removed. For control transplants, rhombomeres were transplanted between the same sides of donor and host embryos, maintaining the rostrocaudal polarity.

way as for donors, leaving the notochord and floor plate in place. In the case of an r3 graft, for example, an r3 from the left-hand side of a donor embryo was rotated through  $180^\circ$  and placed in reverse rostrocaudal polarity in the right-hand side of a host embryo (see Fig. 1). By transplanting from right to left, or vice versa, rostrocaudal polarity was reversed while normal mediolateral and ventricular-pial polarity was maintained. Alternatively, rhombomeres were transplanted homotopically, maintaining their correct rostrocaudal polarity, i.e. by grafting from one side of a donor into the same side of a host embryo. The other side of the hindbrain was left undisturbed as a control. Operated eggs were sealed with tape and returned to the incubator for 48 hours or until they had reached stage 19-21. Embryos in which the transplants had failed to heal adequately at both ends were excluded from analysis. In embryos that were analysed, the operated side of the embryo had the histological and morphological appearance of a normal hindbrain. Grafted tissue maintained its original orientation during subsequent development.

#### *Retrograde labelling of cranial nerves in transplanted embryos*

Embryos in which rhombomere grafts were incorporated into the neuroepithelium were fixed for 2 hours in 3.5% formaldehyde and solutions of fluorescent carbocyanine dyes DiI and DiO (Molecular Probes, Oregon, USA;  $3 \text{ mg ml}^{-1}$  in dimethylformamide) were pressure injected into the cranial nerve roots, just lateral to the brain. In r3 transplants, nerves V (DiI) and VII (DiO) were injected, while in r5 transplants nerves VII (DiO) and IX (DiI) were injected. The contralateral, unoperated sides of these embryos were also injected and analysed in the same way, to serve as controls. Labelled embryos were incubated for 24 hours at room temperature, in 3.5% formaldehyde (Godement et al., 1987). Brains were then dissected out as for immunostained whole-mounts and viewed ventral side up with epifluorescence using green (DiI) or blue (DiI + DiO) excitation.

For each operated embryo, the length of the transplanted

rhombomere was measured using a graticule, and the number of axons originating from cell bodies within this territory was counted. Labelled axons were then apportioned either to the rostral or caudal exit point, since in r3 grafts, for example, axons exiting in r2 were labelled red (DiI) and those exiting in r4 were labelled green (DiO). The length of the grafted segment was measured, and then the length occupied by axons of either colour. For example, in the case of r3 reversals, the territory occupied by red axons exiting in r2 was measured from the r2/r3 boundary to the most caudal axon. The territory occupied by green axons was measured from the r3/r4 boundary to the most rostral axon. Length was calculated as a percentage of the total segment length for subsequent analysis. This method of measurement thus took no account of the density of axons, or of spaces unoccupied by axons.

## **Results**

### *Motor neuron development visualised using DM1 staining*

For the analysis of motor neuron development using whole-mount antibody staining, we have concentrated on three branchiomotor nuclei, those of the trigeminal (V), facial (VII) and glossopharyngeal (IX) nerves, since these were the subject of our study on the effects of reversing rhombomere polarity. There are limitations in interpreting development using a single antibody since it is unclear whether DM1 labels all motor neurons in the nuclei under scrutiny, or only a subset. Therefore, a description of the precise timing of formation and outgrowth of branchiomotor neurons in the hindbrain awaits confirmation using other methods. The immunohistochemistry described here should be considered largely as an adjunct to the transplantation experiments.

Our descriptions of DM1 immunoreactivity in whole-mounts refer to flattened whole-mount preparations, in which the ventral midline lies medial, and the dorsal edges of the neural tube lie lateral. No immunoreactivity was observed in hindbrains prior to stage 13. In late stage 13 and stage 14 embryos, staining was observed in the floor plate in a domain extending from the spinal cord up to the vicinity of the rhombomere 5/6 (r5/6) boundary (Fig. 2A,B). The first immunoreactivity in motor neurons was observed in cell bodies grouped around the r4/5 boundary, and extending about half a segment in either direction rostrocaudally. By stage 15, rhombomere 4 contained the fan-shaped arrangement of axons characteristic of the facial nerve nucleus. A large number of immunopositive cell bodies were also evident in r7, r8 and caudally within the spinal cord (Fig. 2C). Immunopositive cell bodies were far more abundant in r7 than in r6 and outgrowing axons were noticeable in r7 only. In most preparations there were also a few outgrowing axons from neurons in r5. In most stage 15 specimens, the trigeminal nucleus was now labelled weakly; more growth cones had reached their exit point in r2 than in r3 (Fig. 2C).

Between stage 15 and stage 16 there was a large increase in the number of immunopositive motor neurons in the hindbrain with r5 full of axons, and the

trigeminal nucleus stained much more intensely than at stage 15. Rhombomeres 6 and 7 displayed the opposite pattern of development to the pairs of rhombomeres containing the trigeminal and facial nuclei. In the latter, the development of the rostral, even-numbered rhombomere was advanced relative to the caudal, odd-numbered partner in which axon outgrowth occurred in a rostrocaudal sequence. In the case of r6 and r7, however, immunopositive cell bodies and outgrowing axons appeared first in r7 at stage 15, and labelled axons were not seen in r6 until stage 16. Within this rhombomere, axons originating in the caudal part of the segment were seen to have reached the exit point first. This pattern is likely to reflect the development of both the glossopharyngeal and vagal motor nuclei, both of which lie within r6 and r7, but which have their exit points in r6 and in r7 respectively. In the region caudal to the r6/r7 boundary, including r7 and r8, there was uniform staining of outgrowing axons from late stage 15 onwards.

By stage 17, all the hindbrain rhombomeres contained DM1-positive motor neurons (Fig. 2D) including those of the trochlear (IV) nucleus, located in r1, near the midbrain-hindbrain isthmus. In a stage 19 preparation, motor neurons were abundant in all segments, and the colonisation of the boundaries by the transversely growing axons became a prominent feature (Fig. 2E). Specimens of stages 16-19 showed considerable variability in the intensity of staining in the trigeminal motor nucleus. In most preparations trigeminal neurons stained much less intensely, with particularly little staining of cell bodies. This discrepancy could also be seen in parasagittal sections showing rhombomeres 3 and 4, and so was not an artefact of whole-mount staining (Fig. 2H). Therefore, it is impossible to say whether motor neurons develop precociously in r4 based on DM1 staining patterns, since the first motor neurons in r2 might not have been detected. At stage 12, neurogenesis is advanced in r4 relative to r2, notably in the production of reticular neurons (Lumsden and Keynes, 1989), but we cannot tell whether this differential is maintained later in development. The period for which trigeminal motor neurons retained DM1-positivity was also shorter than for facial motor neurons. Between stage 19 and stage 25, immunoreactivity on the proximal segments of the trigeminal motor axons was gradually lost, but was retained in both the ophthalmic and maxillo-mandibular divisions of the trigeminal nerve outside the brain (Fig. 1F). At stage 25, immunoreactivity was evident in the somatic motor cranial nerves (III, IV, VI and XII), and in the branchiomotor (V, VII, IX, X and XI) nerves, but not in the ophthalmic, optic or vestibulo-acoustic nerves (I, II and VIII). Of the DM1-positive cranial nerves, only the trigeminal nucleus showed a substantial down-regulation of the DM1 antigen (Fig. 2F).

Other sites of localisation of DM1 staining were the floor plate and notochord. Both these sites of DM1 binding are evident in a transverse section taken at the level of r5 and the otic vesicle (Fig. 2G). In parasagittal sections, the rostral limit of DM1 staining in the

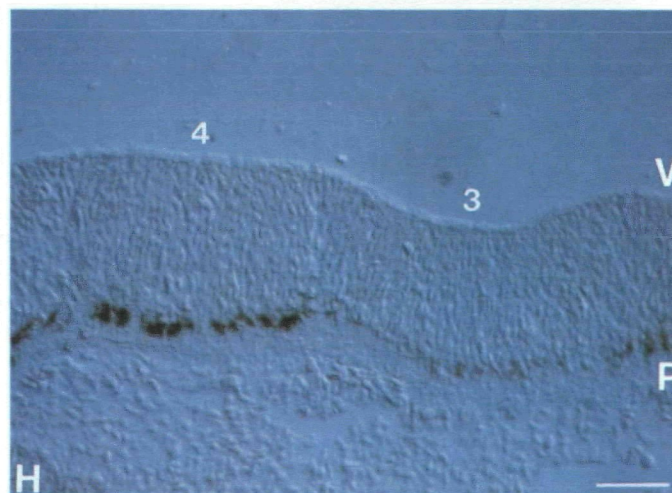
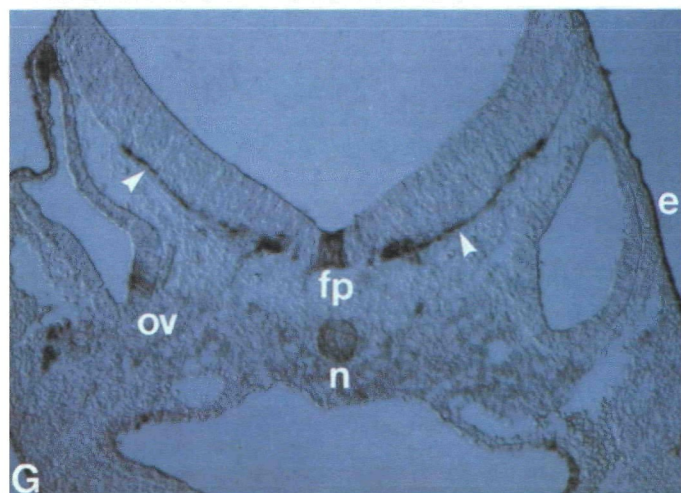
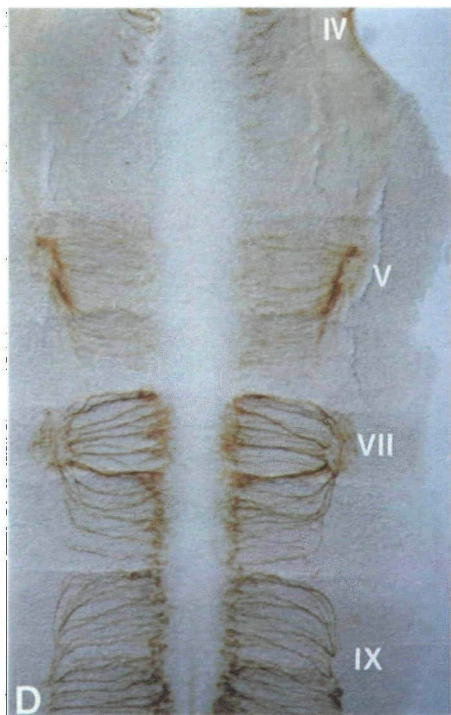
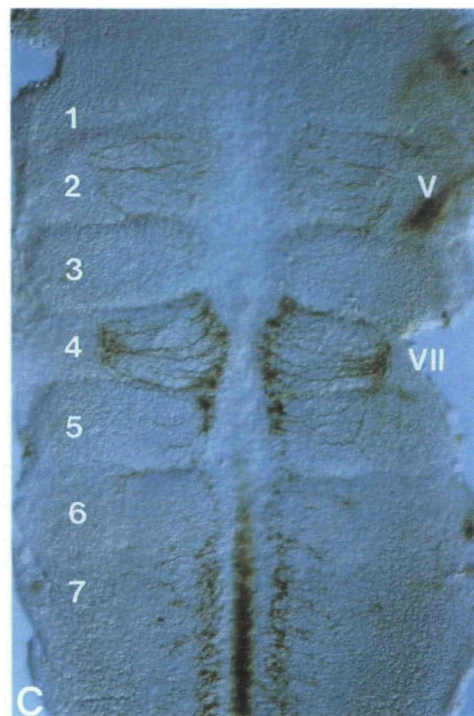
**Fig. 2.** Whole-mount and sectioned hindbrains showing staining of motor neurons in normal embryos. Top of the diagram is rostral. (A) Stage 14. (B) Stage 14, showing localisation of stained motor neuron cell bodies at the r4/5 boundary (arrows). (C-F) Numbers indicate rhombomeres. Roman numerals indicate cranial nerves and are placed adjacent to the nerve roots: III-oculomotor, IV-trochlear, V-trigeminal, VII-facial, IX-glossopharyngeal, X, XI-vagus/accessory. (C) Stage 15. (D) Stage 17. (E) Stage 19. (F) Stage 25; preparation includes cranial nerve roots and sensory ganglia. (G) Transverse section of stage 21 hindbrain showing staining in notochord (n), floor plate (fp), ectoderm (e) and otic vesicle (ov). Arrows indicate motor neurons coursing from their ventral origin laterally (dorsally). (H) Parasagittal section of stage 21 hindbrain showing increased intensity of staining in r4 (facial nucleus) relative to r3 (trigeminal nucleus). V=ventricular surface of neuroepithelium. P=pial surface. Scale bar=100  $\mu$ m for A, C, D, E and G, 40  $\mu$ m for B, 200  $\mu$ m for F, 45  $\mu$ m for H.

notochord was seen to correspond with that in the floorplate at stage 15-17 (not shown). From stage 17 onwards, DM1 staining in the floor plate remains strongest caudal to the r6/r7 boundary, but from stage 17-25 the DM1 antigen becomes increasingly expressed in the medial third of the floor plate in the hindbrain rostral to that point. The significance of this pattern is unclear, except that the notochord is known to have an important role in inducing the formation of the floor plate (van Straaten et al., 1988; Yamada et al., 1991).

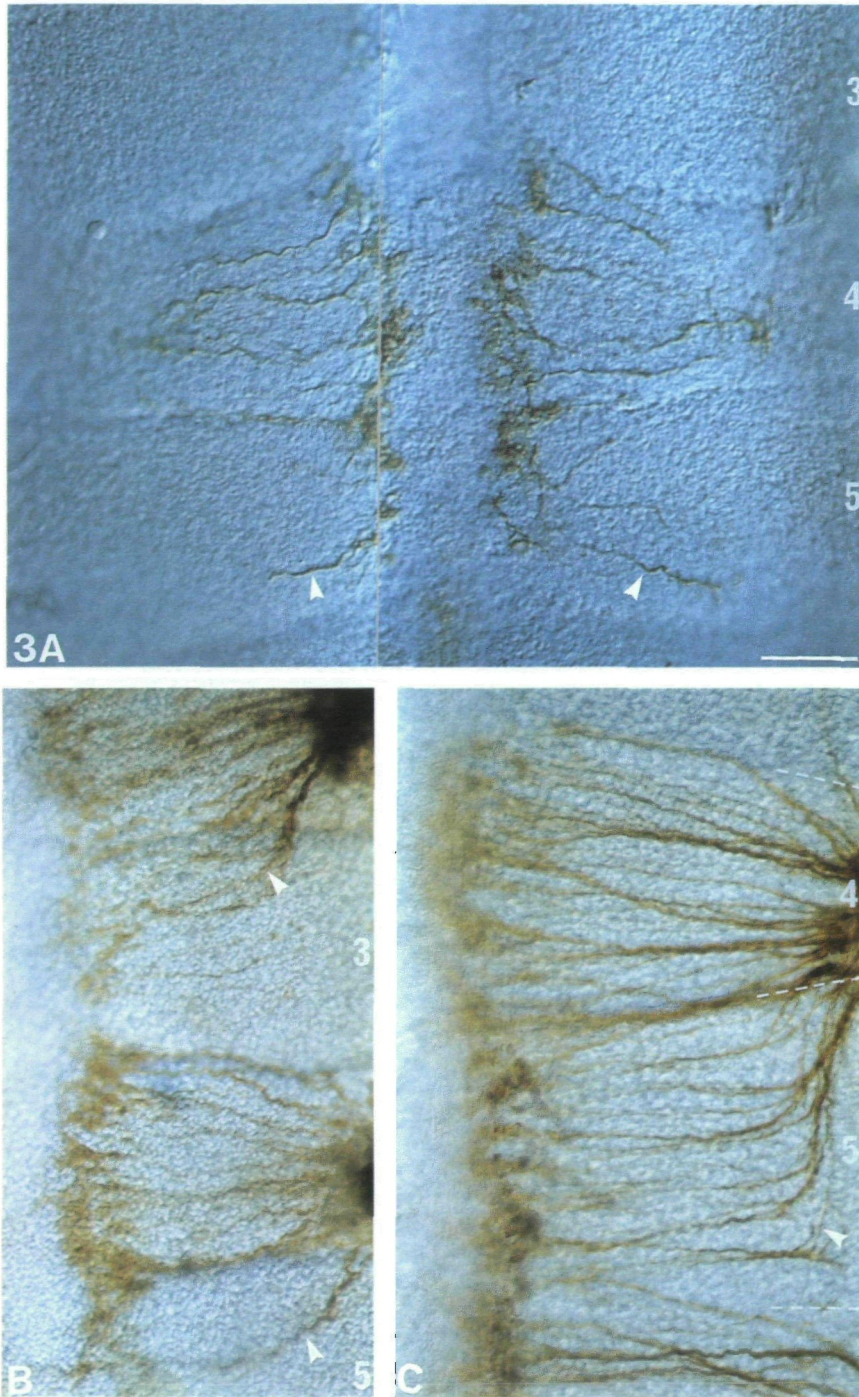
#### *Axonal trajectories in odd-numbered rhombomeres*

In describing motor neuron patterning in normal and manipulated embryos, we assume that motor neurons that lie within r2 and r3 have specific identities as trigeminal neurons, those within r4 and r5 as facial neurons, and so on. Each specified group of motor neurons is matched to its corresponding rostral exit point. Failure of trigeminal or facial axons to exit at r2 or r4, respectively, could then be described as errors. The development of axon trajectories in rhombomeres 3 and 5 presents a particularly interesting problem, since here axons must turn rostrally to reach their exit point. Branchiomotor axons in odd-numbered rhombomeres that grow caudally, or that grow out of the brain via a caudal exit point might thus be considered as incorrect or aberrant. DM1 staining at late stages of development (Fig. 2E,F), and DiI retrograde labelling of cranial nerve roots (Lumsden and Keynes, 1989) shows the apparently stereotyped pathways taken by motor axons in odd-numbered rhombomeres to reach their exit point in the rostrally adjacent rhombomere. The development of these trajectories can be visualised in DM1-stained hindbrains, concentrating on r5, since here motor neurons were stained more intensely than in r3. At stage 19-21, motor axons within r5 formed stereotyped arrays, with parallel axons extending from the floor plate-basal plate boundary towards the dorsal (lateral) edge of the neuroepithelium, before turning rostrally almost at right angles (Fig. 3C). However, these patterns were not foreshadowed at earlier developmental stages, by axons which showed an initial





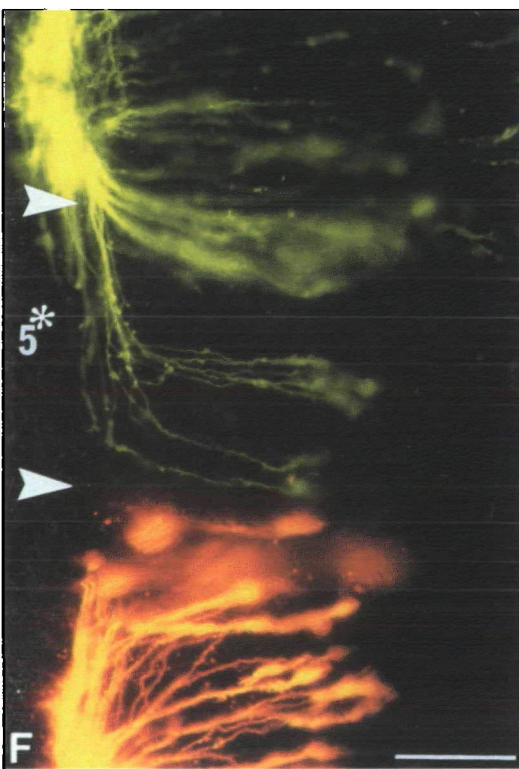
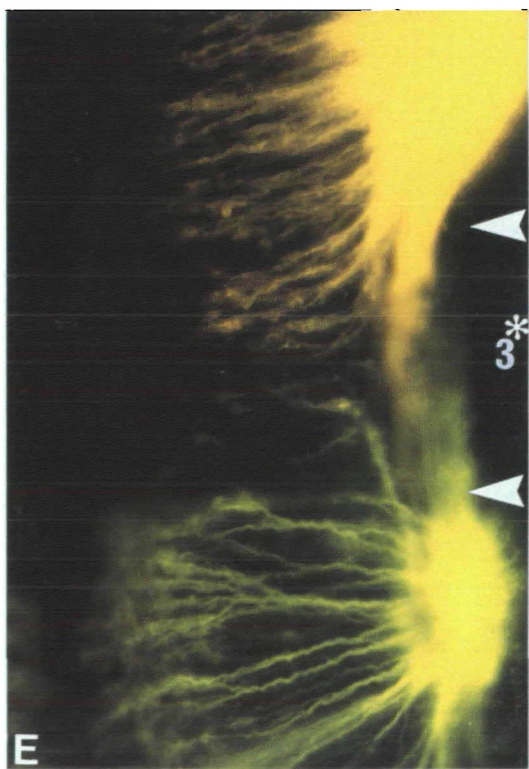
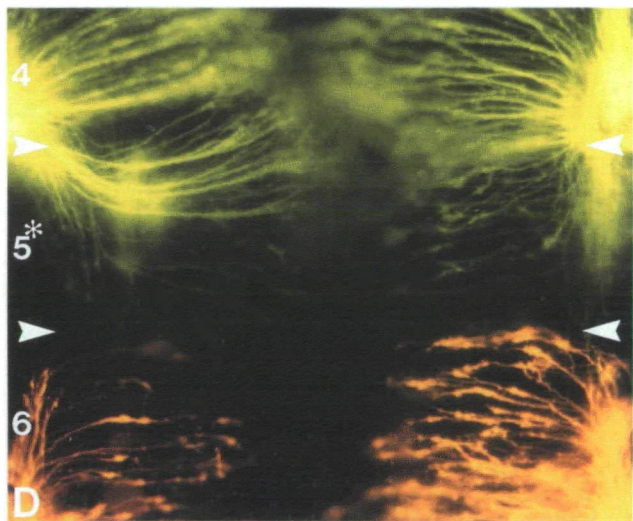
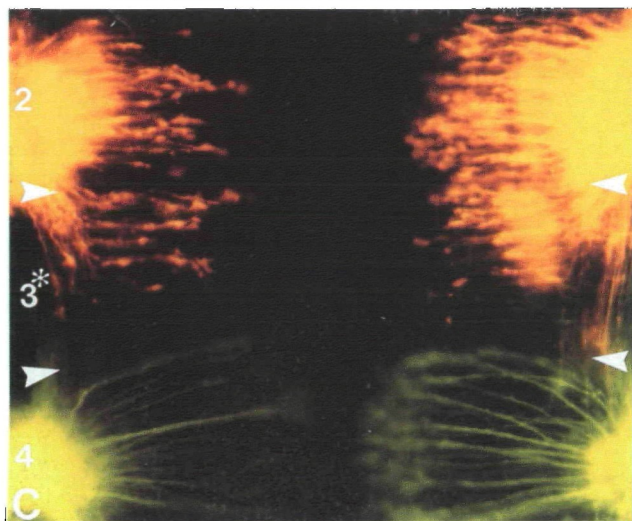
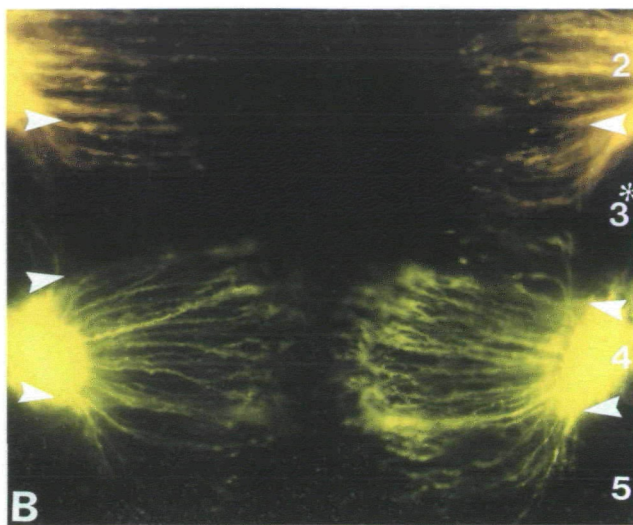
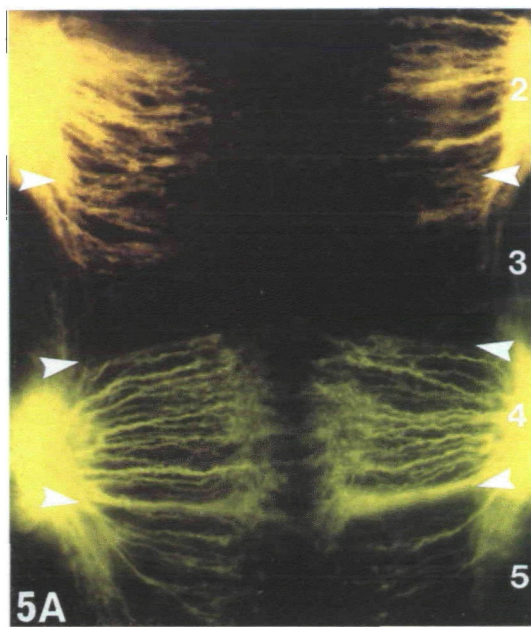




**Fig. 3.** Whole-mount hindbrains showing motor axon configurations at different stages in normal embryos. In all panels numbers indicate rhombomeres. (A) Stage 15; axons are seen growing caudally within r5 on both sides of the embryo (arrows). (B) Stage 16; examples of axons with arcuate trajectories, in r3 and r5 (arrows). (C) Stage 21; axons in r5 turn sharply towards the exit point. Dotted lines indicate rhombomere boundaries. Scale bar=50  $\mu$ m.

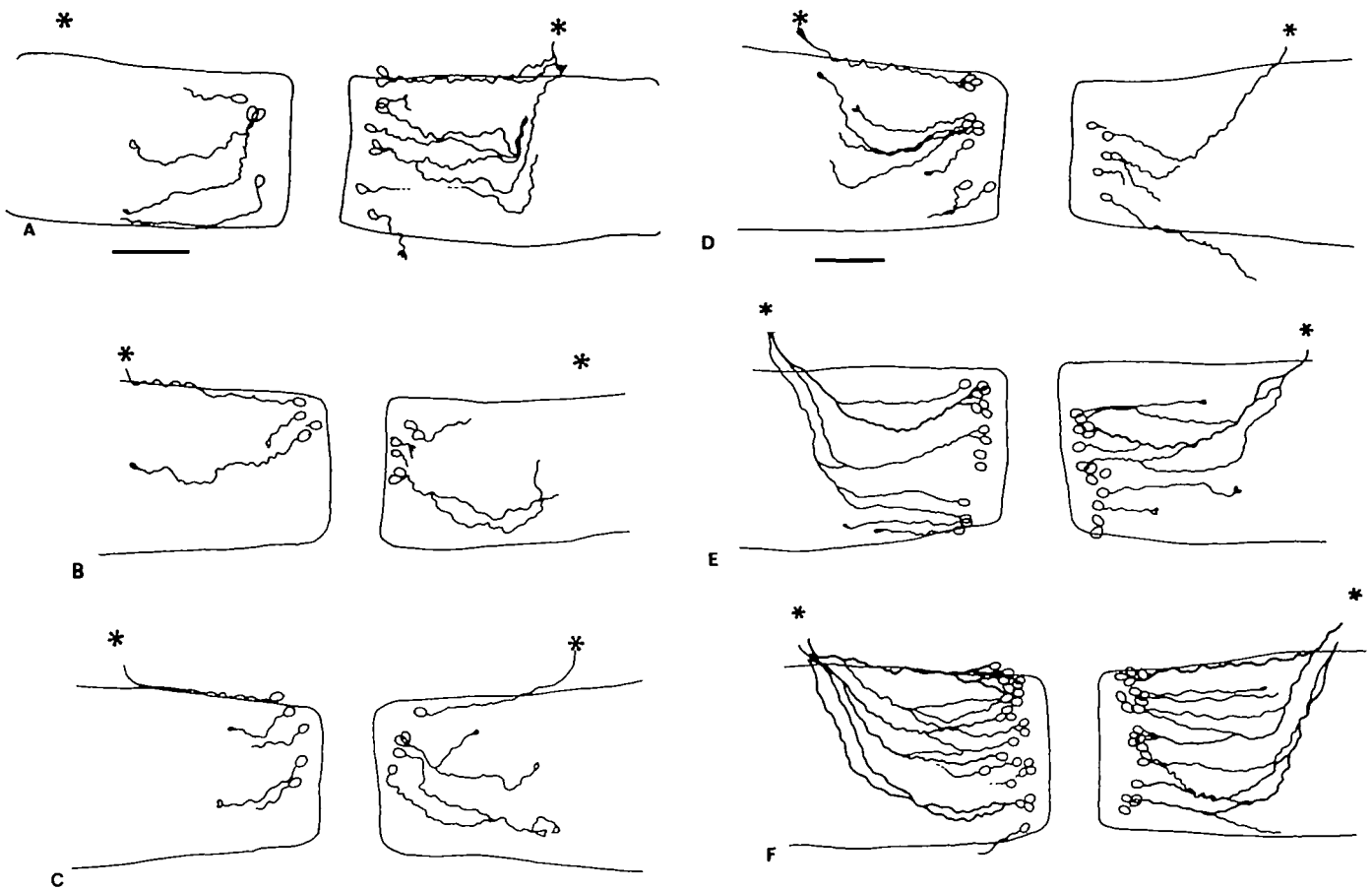
**Fig. 5.** Retrograde DiI/DiO labelling in unoperated embryos, control transplants and rhombomere reversals. Top of the diagram is rostral. In all panels rhombomere boundaries are indicated by arrows, and rhombomeres are numbered. In B-F asterisk indicates transplanted rhombomere. A-D show both operated and control sides of embryos. E and F show only operated side of embryo. (A,B,C,E) Motor neurons in R3 - motor axons labelled via trigeminal (V) exit point yellow or orange (DiI), motor axons labelled via facial (VII) exit point green (DiO). (D,F) Motor neurons in R5 - motor axons labelled via facial (VII) exit point green (DiO), motor axons labelled via glossopharyngeal (IX), orange (DiI). (A) Normal, unoperated embryo showing the majority of axons in r3 exiting in r2 - trigeminal exit point. (B) R3 reversal showing contingent of axons exiting in r4. (C) R3 control transplant in correct orientation showing large majority of R3 axons exiting in r2. (D) R5 control transplant in correct orientation, showing all axons exiting in R4 as on control side. (E) R3 reversal at higher power. (F) R5 reversal, showing similarity to control transplant - all axons from r5 exit in r4. Scale bar=100  $\mu$ m for A-D, 65  $\mu$ m for E and F.











**Fig. 4.** Camera lucida drawings of motor neurons in whole-mount preparations; rhombomere 5. Examples of camera lucida drawings of motor neurons; each right and left pair belong to the same embryo. Top of the diagram is rostral. The outlines of the rhombomeres show the locations of the transverse rhombomere boundaries, and the medial floor plate/basal plate boundary. Stars denote the positions of the centres of rostrally located exit points in r4. Thin lines denote single axons, thicker lines bundles of more than one axon. (A-D) Stage 15. (E) Stage 16. (F) Stage 17. Scale bar in A = 50  $\mu$ m for A-C. Scale bar in D = 50  $\mu$ m for D-F.

phase of this trajectory. At stage 17, even though a considerable number of axons had reached the exit point, they had predominantly arcuate courses (Fig. 3B). Even earlier, at stages 15 and 15+, DM1-positive axons could be seen pursuing a variety of routes. Some appeared to take direct routes to the exit point, but the majority were meandering, and often, some axons could be seen heading caudally, apparently towards the inappropriate exit point in r6 (Fig. 3A). Such axons were also observed in r3.

More detailed analysis of the early trajectories of motor neurons in r5 was possible from camera lucida drawings of DM1 whole-mounts. In Fig. 4A-D, four examples of paired rhombomere 5s at stage 15 are presented. Motor neurons that lay close to or on the r4/5 boundary often had long axons which grew in a fairly straight line along the boundary and then turned rostrally for a short distance towards the VII exit point [Fig. 4B (left-hand side), Fig. 4C (both sides)]. However, neurons whose cell bodies lay within r5, either in the rostral or caudal portion of the segment, often had meandering axons which grew caudally [Fig.

4A,D (right-hand side); Fig. 4B,C,D (left-hand side)]. The frequency of axons growing caudally appeared to be greater among neurons whose cell bodies lay in the posterior third of the segment. At stage 15, the longest axons in the rhombomere appeared to belong to the most rostral cells bodies, implying a rostrocaudal wave of maturation within the segment. At stage 16, more axons had reached the VII exit point, and neurons whose cell bodies lay adjacent to each other were seen to fasciculate (Fig. 4E). One prominent feature of motor neuron outgrowth in r5 was the paucity of pioneers. Outgrowing axons were not evenly spaced, but were bundled together at irregular intervals, tending to fasciculate with neighbours even if this meant taking a more indirect route to the exit point. By stage 17, the majority of labelled axons had reached the exit point (Fig. 4F), and the number of axons growing caudally appeared to be fewer. In DM1-stained preparations at stage 19 and later, axons that had grown to the wrong exit point could sometimes be observed. Based on these observations, it appears that facial motor neuron trajectories are not rigidly determined,



that their axons become realigned during development and that some axons leave the hindbrain via the inappropriate exit point.

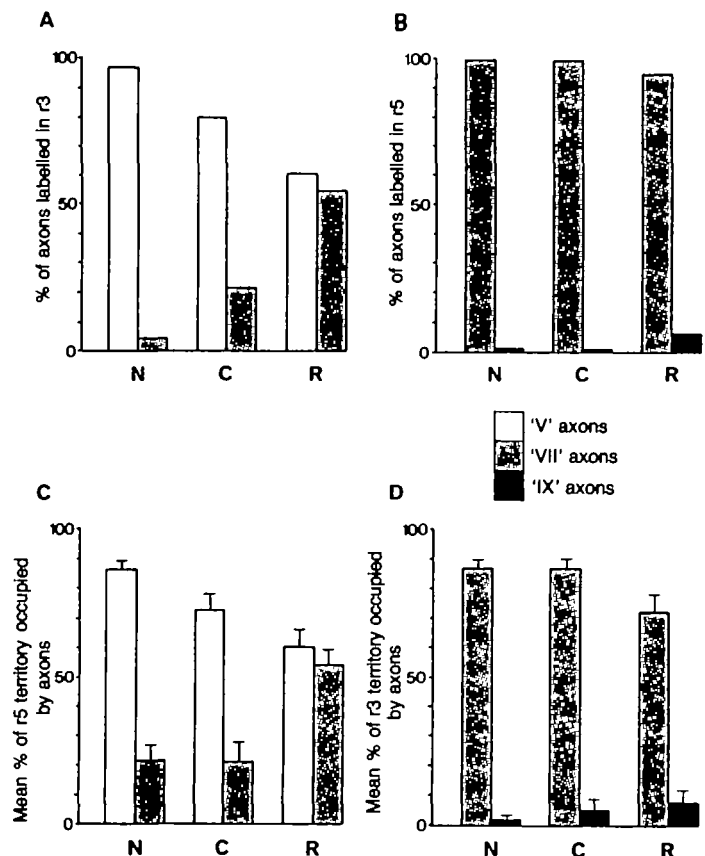
#### *Patterning of motor neurons in embryos with rhombomere reversals*

In embryos in which the rostrocaudal polarity of r3 or r5 had been reversed and the graft had healed adequately, the superficial appearance of the hindbrain and the patterning of rhombomere boundaries was completely normal. In addition, the cranial nerves lateral to the brain displayed normal morphology. Retrograde DiI/DiO labelling was used to visualise the disposition of branchiomotor neurons in these embryos. This method was used since DM1-staining was inadequate for detection of trigeminal motor neurons. However, DiI-labelling allowed us to visualise only those axons that had emerged through the exit point at the time of labelling. For r3 reversal experiments, the mean number of axons exiting the brain in r2 was reduced to 49% of those in normal embryos, reflecting either failure of outgrowth, misrouting or cell death. For r3 transplants in the correct orientation, this figure was somewhat higher (68%). Surprisingly, for r5 transplants, both in the correct and in the reversed polarity, the percentage of outgrowing axons did not differ significantly from that in unoperated embryos.

#### *R3 reversals*

In unoperated embryos injected with DiI/DiO into nerves V/VII, the large majority of motor neurons in r3 constituted part of the trigeminal nucleus, being filled via the exit point in r2 (Figs 5A, 6). However, in 8/12 normal embryos, one or two neurons located in r3 extended axons via the VII exit point. Thus 27/684 neurons (4% of the total number of neurons counted) contributed to a positionally inappropriate exit point, at least up to stage 19-21. In embryos in which r3 had been transplanted in the correct orientation, it was also the case that the majority of embryos (7/10) contained some r3 axons navigating to the VII exit point (Fig. 5C), but the number of these axons as a percentage of the total counted was higher (81/391 or 21%).

In embryos in which r3 had been reversed, there was a dramatic shift in the proportions of axons labelled via the V or VII exit point. If motor axons were specified relative to their particular exit point within the hindbrain, then it might have been expected that, in the reversed rhombomere, they would still course rostrally, toward the exit point in r2. If, on the other hand, they were governed by an intrinsic polarity in the segment, then they would obey this, irrespective of the reversal, and course caudally toward the exit point in r4. In fact, we observed that now in most embryos (14/17), motor axons in r3 split into two contingents, to find their way out either at the V or at the VII exit point; these will be referred to as "V" or "VII" axons (Figs 5B,E, 6). Of all axons counted in all embryos, 277/475 (58%) homed in on the trigeminal exit point, while 198/475 (42%) exited with the facial motor neurons. Thus, the majority found the appropriate exit point despite reversal of the



**Fig. 6.** Histograms showing the percentage of axons apportioned to each exit point, and the amount of rhombomere territory occupied, in normal embryos and rhombomere transplants. Axons labelled via trigeminal (V) exit point are represented by shaded bars, those via facial (VII) exit point by filled bars, and those via glossopharyngeal (IX) exit point by open bars. N=unoperated embryos. C=rhombomeres transplanted in the correct orientation. R=rhombomeres transplanted with reversed rostrocaudal polarity. (A) shows the percentage of axons labelled in r3 which exited via the V or VII exit points in normal embryos and r3 transplants. (B) shows the mean percentage of rhombomere 3 territory occupied by axons taking the V or VII exit points in normal embryos and r5 transplants. (C) shows the percentage of axons labelled in r5 that exited via the VII or IX exit points in normal embryos and r5 transplants. (D) shows the mean percentage of rhombomere 5 territory occupied by axons taking the VII or IX exit points in normal embryos and r5 transplants. Error bars in C and D show  $\pm$ s.e.m.

segment. Assessment of the extent of the segmental territory occupied by these neurons showed that the mean percentage of the segment length occupied by "V" axons (60%) closely mirrored the percentage of neurons taking this pathway, in the same way as for "VII" axons (54%). Even in cases where very few r3 neurons projected caudally, their cell bodies were often located quite far from the r3/r4 boundary. By contrast, in r3 transplants in the correct polarity, "V" axons occupied 72% of the segment and, in normal embryos, this figure was increased to 86% (Fig. 6). In r3 transplants made in the correct orientation, "VII"

axons constituted 21% of the total axons counted and occupied 21% of the segment. Surprisingly, in unoperated controls where only 4% of the total exited with nerve VII, this figure remained 21% of segment length.

#### *R5 reversals*

R5 reversal experiments gave a strikingly different result from r3 reversals. In by far the majority of normal, unoperated embryos (17/20), all of the labelled axons in r5 took the appropriate route via the VII exit point in r6. Only the occasional axon, amounting to 7/682 (1%) of all those labelled, was seen to emerge via nerve IX (Fig. 6). Only 2/12 embryos in which r5 had been grafted in the correct polarity showed any axons finding the IX exit point (Figs 5D, 6), and the number of axons exhibiting this behaviour remained low (3/244 or 1%). In embryos with reversed rhombomere 5s there was only a small increase in the number of "IX" axons. Only 3/13 embryos showed a few aberrant "IX" axons, amounting to 29/455 (6%) of all those labelled (Fig. 6). Thus, in spite of the reversal of the neuroepithelium in which they lay, these axons were able to navigate to their appropriate exit point, in a direction that was caudal relative to the original neuroepithelial polarity (Fig. 5F).

When the extents of the territories occupied by the "VII" and "IX" axons are compared between normal embryos, r5 transplants and r5 reversals, there is little difference. In unoperated embryos, the mean length occupied by "IX" axons was 2% of the total segment length, while 5% was the mean percentage length occupied in transplants in normal polarity, 8.5% in reversals. The mean percentage length occupied by "VII" axons was 87% for both unoperated embryos and transplants in the correct polarity, 72% for reversals.

A summary of the results obtained in r3 and r5 transplants is presented in Fig. 6. For statistical analysis we considered the null hypothesis that in each embryo 90% or more axons should exit rostrally and counted the proportion of embryos in each experimental category to which this applied. For r3 experiments, the proportion of unoperated embryos was then compared with control transplants using the  $\chi^2$  test and found not to differ, while the difference between the distribution of embryos in reversal experiments differed from that in controls significantly ( $P < 0.01$ ). For r5 transplants neither the control transplants nor the reversals differed significantly from the unoperated embryos, and the same result was obtained even when the null hypothesis was altered such that 100% of axons were expected to exit rostrally. This suggests that in the case of r5 transplants, the mean figure of 6% for axons exiting caudally gives a false impression, since in the large majority of reversals (10/13), 100% of axons exited rostrally. The percentage of the segment length occupied by each group of neurons and the area of overlap is shown for individual embryos in Fig. 7. No account is taken here of the density of neurons, so the analysis tends to exaggerate the significance of the territory occupied by the aberrant axons. However, these results serve to illustrate that, in some cases of r3 reversals, it

was possible for axons to find an exit point when they originated from cell bodies positioned more than 50% of the segment length away from it, and there was often quite extensive overlap in the territory occupied (Fig. 7C). In contrast, in r5 reversals, there were very few cases of overlap in the territories occupied by "VII" and "IX" axons (Fig. 7F).

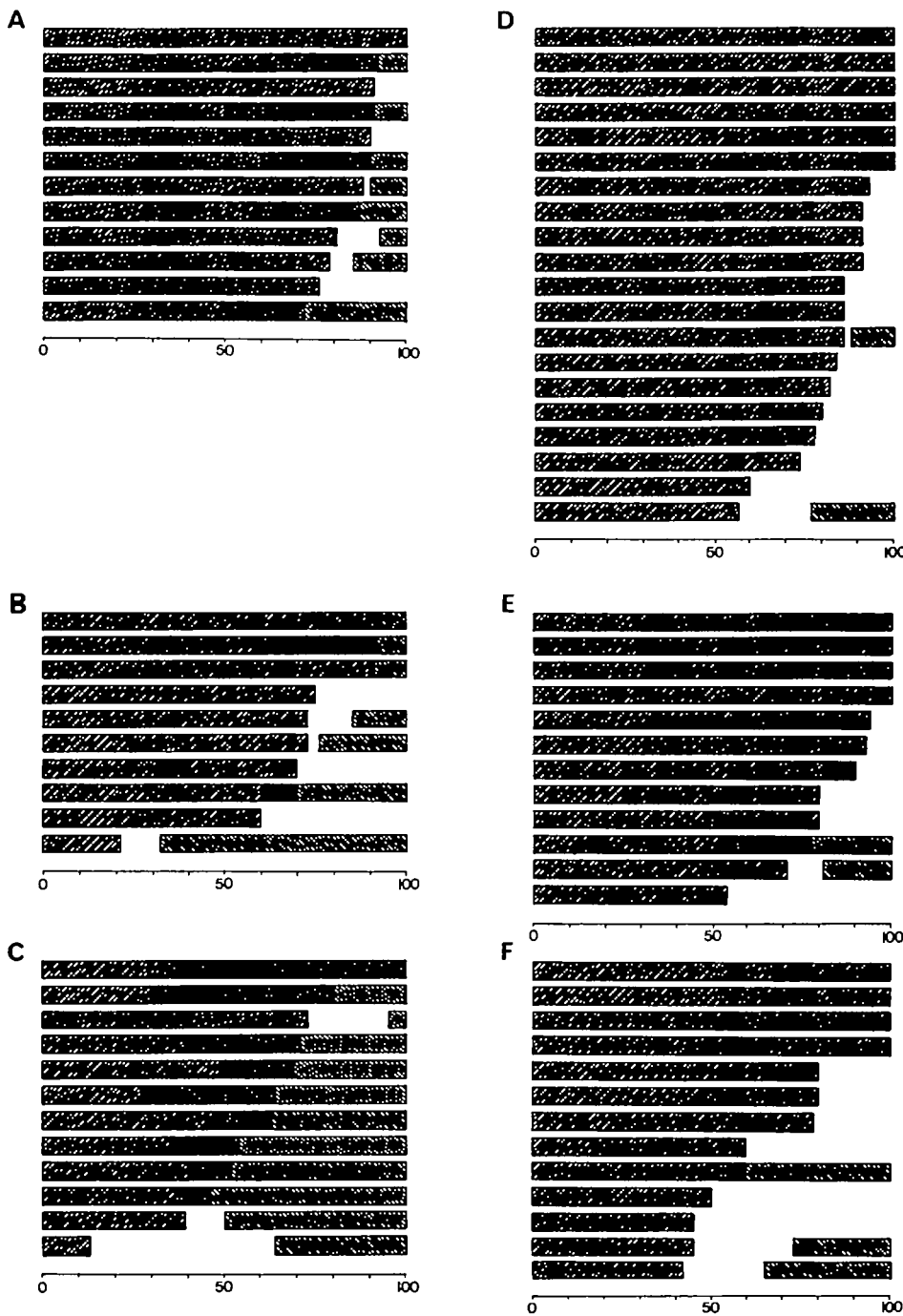
#### Discussion

Analysis of the early development of branchiomotor neurons in the hindbrain has shown that motor axons in odd-numbered rhombomeres do not grow directly towards their rostral exit points, suggesting that their initial guidance is independent of their exit point. Yet following the reversal of normal rostrocaudal polarity in odd-numbered rhombomeres, the majority of motor axons found their correct exit point. This suggests that motor neuron trajectories are not determined purely by distributed positional cues in the neuroepithelium. Instead, branchiomotor growth cones may be attracted by their intermediate targets, the exit points.

Examination of the early trajectories of motor neurons in odd-numbered rhombomeres showed that axons lying on or close to rhombomere boundaries grew along them, reflecting the specialised properties of this region of the neuroepithelium (Lumsden and Keynes, 1989; Guthrie et al., 1991). However, many axons elsewhere within the rhombomere had meandering paths, often with an initial phase of caudally directed outgrowth. Subsequently, most of these axons turned rostrally towards the exit point. Established axonal configurations altered later in development to give the impression of highly stereotyped pathways with axons extending straight laterally, and making sharp rostral turns towards their exit point. In their pathway, axons are threaded between the endfeet of columnar radial epithelial cells. Since these cells withdraw their basal endfoot during each cell cycle, this process might enable the trajectories of axon cylinders to become readjusted. In addition, changes in axonal configurations might be effected by expansion of the neuroepithelium during development.

A common finding in normal embryos was that a small minority of axons in odd-numbered rhombomeres projected via an inappropriate, caudal exit point. Such apparent errors could arise in three possible ways. First, a cell born in r4 and destined to become a facial motor neuron might translocate into r3. Since lineage analysis has shown that rhombomere boundaries restrict cell movement between rhombomeres (Fraser et al., 1990) at least up to stage 19, such translocation might only occur after this developmental stage. Motor axons cross boundaries, however, suggesting either that axons are exempt from this restriction, or that the restriction does not operate in the marginal layer. Secondly, a cell in the caudal part of r3 might be incorrectly specified as a facial motor neuron. In both these cases, caudal growth of axons via the facial nerve exit point would be appropriate to the cell's identity. Thirdly, caudal





**Fig. 7.** Bar diagrams showing the territory occupied by different groups of axons in normal embryos and rhombomere transplants. (A) rhombomere 3, normal embryos.  $n=12$ . (B) rhombomere 3, control transplants.  $n=10$ . (C) rhombomere 3, reversals.  $n=12$ . (D) rhombomere 5, normal embryos.  $n=20$ . (E) rhombomere 5, control transplants.  $n=12$ . (F) rhombomere 5, reversals.  $n=13$ . Each bar on each diagram represents a single embryo. The x axis represents percentage of rhombomere length, with rostral to the left and caudal to the right. Bars represent the percentage of segment length that was occupied by V or VII axons (r3 transplants) or VII or IX axons (r5 transplants). Thus for A, B and C the bars that extend from the left-hand side of the diagram represent axons exiting via the V exit point, while those that extend from the right-hand side of the diagram are those taking the VII exit point. In the case of diagrams D, E and F, these are respectively VII and IX neurons. Rhombomeres from individual embryos have been arranged in order, with those showing the most extensive territory occupied by axons exiting rostrally (V axons for r3 reversals, VII axons for r5 reversals) placed at the top of each diagram, those showing the least territory at the bottom. The area of overlap of groups of neurons is represented by cross-hatching. In cases where bars do not meet, there was a gap between territories occupied by the two groups of neurons.

growth might arise due to true errors of axon navigation, perhaps indicating the multiplicity of factors that influence growing axons.

After segment polarity reversal, the majority of axons still found their way to the correct exit point. Thus the rostrally directed trajectories of motor neurons in odd-numbered rhombomeres are not determined exclusively by polarity cues in the neuroepithelium. Instead, it is possible that the exit point, or some component related to it, may be a source of specific guidance cues. Either branchiomotor neurons may be attracted to their appropriate exit point and

specified with respect to it, or motor neurons may be attracted to exit points in general. At present we cannot distinguish between these possibilities, but the evidence from reversals suggests that although motor neurons can utilise the positionally inappropriate exit point the majority prefer to grow to the appropriate exit point, given the choice. The idea of motor neurons growing to the nearest available exit point is attractive because of its simplicity, but it is hard to envisage how this would work in normal development where the motor neurons in the caudal halves of r3 and r5 are closer to their caudal exit point. Normal patterns could then only be

explained by a timing mechanism that matched the maturation of motor neurons in a particular nucleus with the maturation of their exit point.

In r3 reversals, the projection of some caudal (originally rostral) axons via the inappropriate, caudal, exit point may reflect a response to neuroepithelial polarity, with axons following their normal rostral course to end up caudal within the reversed environment. The same behaviour is not displayed by rostral (originally caudal) axons, possibly because the effect of segment polarity is overridden by an interaction with the trigeminal exit point. Alternatively, there might be a rostrocaudal gradient of maturation of motor neurons within r3. In reversed segments, rostral neurons which end up caudal may be amenable to an influence from the r4 exit point, during a responsive period in their maturation. To explain the patterns of normal development, the attractive influence of the r4 exit point would have to subside by the time the caudal r3 motor neurons were responsive. Immunostaining of motor neurons within r3 and r5 did give some grounds for suspecting a rostrocaudal wave of maturation (Fig. 4).

In r5 reversals, over 90% of axons showed homing behaviour, compared with 58% in r3 reversals, suggesting that r5 axons may have a more specific affinity for their appropriate exit point. Another contributory factor may be that the IX exit point lies towards the caudal part of the rhombomere 6 (Figs 2F, 5D), whereas both the V and VII exit points lie in the centre of their respective segments. If motor axon outgrowth orients towards a diffusible signal from any exit point, the caudal location of the IX exit point would explain why fewer motor neurons in r5 grow caudally relative to those in r3, either in normal, control operated or segment-reversed embryos. Another reason for the difference between r3 and r5 reversals may lie in the timing of axonogenesis of motor neurons in these rhombomeres relative to the formation of their exit points.

The origin of the exit point cells is not known. In the trunk, the ventral root sheath cells are derived both from the neural crest and from cells located more ventrally within the neural tube (Lunn et al., 1987). Certainly the trigeminal ganglion (which is partly derived from the neural crest) influences the development of the trigeminal motor nucleus. When trigeminal ganglia were ablated, the number of trigeminal motor axons exiting the neural tube was severely reduced (Moody and Heaton, 1983c), and the mediolateral translocation of their cell bodies was inhibited (Moody and Heaton, 1983a,b). Sensory axons extend into the hindbrain, where they form lateral longitudinal fasciculi, which may later provide contact guidance pathways for motor neuron growth. However, the initial phase of axonal outgrowth occurs without recourse to these pathways. A possible chemotropic influence could emanate either from the sensory neuron component of the ganglion, or from satellite cells. As yet we do not know which cells are responsible for breaching the basal lamina of the neural tube and establishing the exit points.

Chemotropism may be involved in several components of the developing nervous system, notably in the innervation of the maxillary process by trigeminal sensory neurons (Lumsden and Davies, 1983; 1986), the initial pathway of spinal cord commissural axons towards the floor plate (Tessier-Lavigne et al., 1989), and in the corticopontine projection (Heffner et al., 1990). A subpopulation of motor neurons in the trunk, the epaxial motor neurons, show target attraction to the dermomyotome (Tosney, 1987), although a chemotropic effect of the dermatome remains to be demonstrated *in vitro*.

Experiments on the optic pathway suggest that axon guidance involves the operation of both local and distributed pathfinding cues. In amphibia, retinal axons from transplanted eyes show homing behaviour towards the optic tectum (Harris, 1980; 1986), though such directed growth fails to occur *in vitro* (Harris et al., 1985), and removal of the tectum does not result in misrouting of axons (Taylor, 1990). Following the rotation of small pieces of the optic pathway, outgrowing retinal axons turned in a direction corresponding with the rotation of the neuroepithelium, suggesting a distributed system of positional information (Harris, 1989). The optic pathway also contains a pre-existing population of axons, the postoptic commissure, which could provide a substratum for retinal axon outgrowth (Easter and Taylor, 1989). Evidence for pathfinding within the CNS has also come from studies in which DRG sensory neurons in dorsoventrally rotated spinal cord take abnormal routes to reach their normal pathway, the dorsal column (Holder et al., 1987).

In the chick limb, motor axons could navigate back to their target muscles after dorsoventral wing reversals (Ferguson, 1983), anteroposterior wing reversals (Stirling and Summerbell, 1985), 3-4 segment lumbar spinal cord reversals (Lance-Jones and Landmesser, 1980), and 1-3 segment limb shifts (Lance-Jones and Landmesser, 1981). The factors governing motor axon outgrowth into the limb may involve non-specific highways for the initial phase of the trajectory, in addition to which local cues ensure correct connectivity of motor neuron and muscle. Similarly, in the hindbrain region, motor axons leave the brain and grow through the adjacent sensory ganglia, whose sensory neurons already provide a bridge to the hindbrain. An important difference between the head and the trunk, however, may be that the paraxial mesoderm in the head region does not play a substantial role. In the trunk, the sclerotome is divided into rostral and caudal parts (Keynes and Stern, 1984), and only the rostral sclerotome furnishes a permissive environment for motor axon outgrowth (Rickmann et al., 1985). Caudal sclerotome cells show enhanced levels of binding of peanut agglutinin (Stern et al., 1986), and cause growth cone collapse (Davies et al., 1990). The inhibitory property of the caudal sclerotome thereby imposes segmentation on the motor axons which sprout uniformly down the length of the neural tube. Though segmentation of the cranial paraxial mesoderm has been described in newt and turtle embryos (Jacobson



and Meier, 1984; Meier and Packard, 1984), in the amniote it remains elusive (Anderson and Meier, 1981; Meier, 1981), and no biochemical heterogeneity has been described. Instead, the motor outflow appears to be intrinsically segmented.

Specific matching of cranial motor neurons and targets remains an attractive prospect, both in terms of intermediate (proximal) targets, i.e. exit points, and ultimate (distal) targets i.e. arch muscles. Preferential growth of trigeminal motor axons towards trigeminal exit point cells, for example, might be evident *in vitro*. In this study, we have used the SC1/DM1 antigen purely as a marker, and its possible role in cranial motor neuron development remains to be explored, though *in vitro* studies suggest that purified DM1 protein supports neurite extension from chick sensory neurons (Burns et al., 1991). The contrasting behaviour of r3 and r5 motor neurons in rhombomere reversal experiments suggested there might be differences in the matching of motor axons from different motor nuclei with their targets.

What happens to the motor neurons that fasciculate with inappropriate neighbours, and presumably navigate to inappropriate targets? Are their cell bodies eventually incorporated into the wrong cranial nerve nucleus, or do they die? When sections of the lumbar spinal cord were transplanted rostrally, or reversed rostrocaudally, motor neurons innervated inappropriate muscles without altering their original activation patterns (Landmesser and O'Donovan, 1984). Whether this would apply for cranial motor neurons is not known. Various interesting questions surround the inappropriately projecting motor neurons in embryos with r3 reversals. Amongst those that we intend to address are how long these cells persist in development, which target muscles they innervate, and whether they acquire appropriate descending connections.

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